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On May 9, 2016 the contract period ended after a 1-year no-cost extension. This report summarizes the entire study.

14. ABSTRACT
In this report we integrated the major results from our rat hemorrhagic shock model with the mechanistic details of protection from ischemia and reperfusion injury in hibernating mammals. Arousal from torpor during hibernation occurs rapidly, but there is no evidence of brain injury accompanying this extreme physiological transition. Production of the antioxidant melatonin accompanies arousal, suggesting that it plays a protective role at this time. We investigated the mechanism of melatonin receptor-mediated protection in the brain of the hibernating ground squirrel. Our portable low-volume therapy for severe blood loss is based on hibernation physiology and increases survivability of lethal hemorrhagic shock in rats. This small-volume (1 ml/kg) resuscitation fluid has three main components: 4 M D-stereoisomer of beta-hydroxybutyrate (BHB), 43 mM melatonin, and 20% DMSO. Results from our rat experiments have been translated to a pig model of hemorrhagic shock. Overall these experimental findings have resulted in three patents from the U.S. Patent Office over the course of this study (Patent No. 8,728,532, May 20, 2014; Patent No. 9,149,450, October 6, 2015; Patent No. 9,186,340, November 17, 2015). These findings will be presented at a Pre-IND Meeting of the FDA (PIND 130671) on July 8, 2016.

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Table of Contents

	Page
Introduction……	**4**
Body……	**5**
Key Research Accomplishments……	**7**
Reportable Outcomes……	**9**
Conclusion……	**12**
References……	**15**
Appendices……	**19**
Table 1……	**19**
Table 2……	**19**
Table 3……	**20**
Figure Legends……	**21**
Figure 1……	**24**
Figure 2……	**25**
Figure 3……	**26**
Figure 4……	**27**
Figure 5……	**28**
Figure 6……	**29**
Figure 7 (Schwartz et al. Fig. 3)……	**30**
Figure 8 (Schwartz et al. Fig. 4)……	**31**
Figure 9 (Schwartz et al. Fig. 5)……	**32**
Figure 10 (Schwartz et al. Fig. 6)……	**33**

Introduction

We previously published a small-volume (1 ml/kg) resuscitation fluid based on hibernation physiology that has three main components: 4 M D-stereoisomer of beta-hydroxybutyrate (BHB), 43 mM melatonin, and 20% DMSO (10). Only one concentration of each component was originally tested. In this study we worked towards optimization of this fluid to enhance survival in a rat model of hemorrhagic shock. We also investigated mechanisms of melatonin receptor-mediated protection in the brain of the hibernating ground squirrel. Arousal from torpor during hibernation occurs rapidly, but there is no evidence of brain injury accompanying this extreme physiological transition. Production of the hormone melatonin accompanies arousal, suggesting that it plays a protective role at this time. Melatonin is used as an antioxidant in our small volume resuscitation fluid.

Approach: Two separate dose-ranging studies were conducted for BHB and melatonin in animals with 60% blood loss. BHB was administered at either 4 M, 2 M, or 0.4 M concentration in conjunction with 4.3 mM melatonin and 10% DMSO. Subsequently, melatonin was administered at either 4.3 mM, 0.43 mM, 0.0043 mM, 0.000043 mM, or 0 mM concentration in conjunction with 4 M BHB and 2% DMSO.

We examined how melatonin receptor signaling contributes to protection in the brain during arousal from torpor in hibernating ground squirrels by specifically examining 1) evidence of apoptotic and survival signaling and 2) mitochondrial function and integrity after administration of the competitive melatonin receptor antagonist luzindole.

Results: Ten-day mean survival showed a dose-dependent trend: rats survived longer with higher concentration of infused BHB (4 M BHB, 7.38 ± 1.75 days; 2 M BHB, 5.25 ± 2.22 days; 0.4 M BHB, 2.07 ± 2.05 days). Administering 4 M BHB without melatonin resulted in low mean survival times (4.38 ± 1.42 days). All treatments containing both 4 M BHB and melatonin, regardless of melatonin concentration, resulted in mean survival times of ~7.5 days.

In a separate study, we administered the competitive melatonin receptor antagonist luzindole (30 mg/kg, i.p.) to ground squirrels at the end of a torpor bout, triggering an arousal. We found that luzindole treated animals exhibited caspase-3 activity two times higher than vehicle-treated animals (P=0.01) in the hypothalamus at mid-arousal, suggesting that melatonin receptor signaling is important for protection in this brain region. We also found a 30% decline in succinate-fueled mitochondrial respiration in luzindole-treated animals compared to vehicle-treated animals (P=0.019), suggesting that melatonin receptor signaling is important for optimal mitochondrial function during arousal from torpor.

Conclusions: In the rat hemorrhagic shock model there is a dose-dependent trend in which higher BHB concentration resulted in higher percent survival over 10 days. Melatonin provides therapeutic effects at very low concentrations evident by survival when administering a solution containing 10^6-fold lower melatonin than previously published (10). Melatonin is essential for survival since 4 M BHB without melatonin had a considerably reduced survival rate.

The work with the hibernation model provides evidence for the importance of melatonin receptor signaling in neuroprotection and promotion of optimal brain function in hibernating mammals arousing from torpor. The protective effects of melatonin have been shown in many disease models, but this study provides an example of melatonin's protective role in a natural system and provides important groundwork for future studies involving the mechanism of action of the low-volume therapy for severe blood loss.

Body

Every person in the world is at risk of trauma regardless of social standing, race, religion, or political ideology. Five million people have trauma-related deaths worldwide every year. In the US, traumatic injury is the main cause of death amongst individuals ages 1 to 44. In the year 2000, trauma cost $117 billion in medical care; 10% of the total US medical expenses (1).

Hemorrhage is a problem of major importance in traumatic injury. Hemorrhagic shock is the leading cause of death and complications after trauma, both military and civilian (2). It is also the leading cause of preventable deaths since trauma casualties often occur due to an inability for victims to access medical facilities in a timely manner (3). It is clear that effective control of hemorrhage, and the development of more efficient resuscitation strategies, can save lives (4).

<u>Avoidance of ischemia/reperfusion injury in hibernators</u>

Natural hibernation in mammals is not accompanied by a massive loss of blood due to hemorrhage; however, it exemplifies an exceptional physiological state characterized by a reduction in cardiac output and blood pressure comparable in magnitude to hemorrhagic shock. Furthermore, classic hibernation patterns include multiple days of greatly reduced blood flow resulting from heart beat reduction to 3-10 bpm when animals are in torpor. These hypothermic torpor bouts are regularly interrupted with brief periods of normothermia (37°C) and normal

heart rates of 300-400 bpm (5). This dramatic change in animal physiology resembles ischemia/reperfusion events seen in non-hibernating mammals, however the brain and other tissues of hibernators are protected from the pathology of ischemia (6) and reperfusion injury (7). Such protection is achieved by the employment of an array of inherent adaptations present in the hibernating animal (8).

Here, we investigated the protective effect of melatonin receptor signaling in the thirteen-lined ground squirrel brain by administering luzindole, a competitive melatonin receptor antagonist, upon arousal from torpor when circulating melatonin levels rise naturally. The rapid and extreme transition in cerebral blood flow from torpor to arousal is arguably ischemia/reperfusion-like, however, the hibernator exhibits no evidence of brain damage (9). This study purposefully manipulates a natural system known to have endogenous protective mechanisms, with the goal of gaining some insight into the control of natural neuroprotection.

BHB/M blood loss therapy

In 2010 our laboratory developed a small-volume resuscitation fluid based on hibernation physiology composed of the D-stereoisomer of β-hydroxybutyrate (BHB) and melatonin referred to as BHB/M (10). BHB/M was developed in a rat model of massive blood loss with the goal of providing a portable fluid that would expand the window of opportunity (aka "golden hour") for transport to medical facilities and long-term survival after blood return. We found after 1 hour of 60% blood loss, 4% fluid replacement with BHB/M significantly prolonged survival up to ten days post-blood return compared to control solutions (10). The same solution has been tested in a porcine model of hemorrhagic shock where a survival benefit was also observed (11). However, in both the small and large animal study, only one concentration of each of the components of BHB/M was tested – 4 M BHB, 43 mM melatonin.

BHB/M is highly portable as it is administered in very small volumes (1 ml/kg) and has the potential for self-administration. This is of particularly importance in the military setting where trauma resulting in blood loss can occur in remote locations. In the Korean War, evacuation time of the severely wounded was 3 hours; in the Vietnam War, 83 minutes (12). These times are still outside of the scope of the current "golden hour" for hemorrhagic shock.

For that reason, there is still a need for the development of a resuscitation strategy that will allow injured soldiers to survive for a sufficiently long period to gain access to proper medical care.

The objective of the work presented here is to enhance survival in a rat model of hemorrhagic shock by optimizing the composition and delivery of BHB/M. This work also investigated the effect of the competitive melatonin receptor antagonist luzindole on the physiological parameters of arousal from torpor, regulation of survival and apoptotic signaling, and optimal mitochondrial function in a hibernating mammal during arousal.

Key Research Accomplishments

Comparison of two different therapy delivery protocols

Two groups of animals were treated identically (Figure 1) except one group received a continuous infusion of therapeutic solution beginning after 60% blood loss. No statistical differences ($p>0.05$) were observed in 24-hour survival when comparing the single Bolus Only (mean survival 496.67 ± 314.59 min. n=6) to the single Bolus followed by Slow Infusion (mean survival 149.20 ±142.71 min. n=5) protocol (Figure 2). Based on these results, subsequent studies designed to optimize composition used a single bolus only (Figure 3). All sham-operated animals lived until the experimental end point of 10 days.

Optimization of Therapy Composition

The BHB/M therapy reported previously was developed for hemorrhagic shock treatment and its composition was based largely on animal survival. The objective here was to optimize the composition based on empirical evidence using the hemorrhagic shock model shown in Figure 3.

Melatonin Dose-Ranging Study I

The formulation of BHB/M containing 4 M BHB with 43 mM melatonin in 20% DMSO (n=6) was compared to a solution containing 4 M BHB, 4.3 mM melatonin and 10% DMSO (n=6). Survival curves (Figure 4) were compared 24 hours and 10 days after 60% blood loss.

24-hour survival showed no statistical differences (p>0.05*)* between BHB/M (mean survival 21.0 ± 2.74 hrs) and 4 M BHB, 4.3 mM melatonin and 10% DMSO (mean survival 21.0 ± 2.74 hrs). There were also no statistical differences (p>0.05*)* in survival at 10 days (6.4 ± 2.0 days and 7.4 ± 1.8 days, respectively; Table 1).

BHB Dose-Ranging Study

BHB concentrations of 4 M (n=6), 2 M (n=5) and 0.4 M (n=5) were compared 24 hours and 10 days after 60% blood loss (Figure 5). Table 2 summarizes pairwise comparisons results. In short, at 24 hours, the 0.4 M BHB treatment had statistically lower survival (p<0.05) than the 4 M BHB and the 2 M BHB treatments. At 10 days, only the difference between 0.4 M and 4 M BHB was upheld (p<0.05). However, 10-day mean survival showed a dose-dependent trend where the higher concentrations of BHB resulted in longer survival (4 M BHB, 7.4 ± 1.8 days; 2 M BHB, 5.3 ± 2.2 days; 0.4 M BHB, 2.1 ± 2.1 days).

Melatonin Dose-Ranging Study II

Survival curves for the concentrations of melatonin shown in Figure 6 were compared 24 hours and 10 days after 60% blood loss. Pairwise comparisons are summarized in Table 3. No treatment differences were observed at either 24 hours or 10 days after 60% blood loss. However, at 10 days, only the treatments with 0 mM melatonin (4.4 ± 1.4 days), 0.0043 mM Mel (6.6 ± 1.6 days), and the NaCl control (4.6 ± 1.4 days) were different (p<0.05) from the sham group.

Melatonin receptor signaling promotes neuroprotection in hypothalamus

In this work, we show an increase in caspase-3 activity in the hypothalamus during mid-arousal with luzindole treatment (Figure 7), indicating that melatonin receptor signaling is important to help quench apoptotic signaling during this time. Previous work showed that melatonin reduced caspase-3 activity through receptor-mediated mechanisms in cultured cells after induction of apoptosis (13, 14). Another study showed that melatonin was protective against cytotoxicity in cultured cells, indicated by a reduction in caspase-3 activation and an enhancement in STAT3 phosphorylation (15). Luzindole attenuated these protective effects in

both cases, indicating that melatonin receptor signaling played an important role in orchestrating the protection. While we found no effect of luzindole treatment on levels of phosphorylated STAT3 (Figure 8), our mid-arousal caspase-3 activity results support this previous work. Interestingly, there is no longer a difference in caspase-3 activity between treatment groups in the hypothalamus at the full arousal endpoint (Figure 7). Overall caspase-3 activity levels are lower in the hypothalamus at the full arousal point, which is at the end of the physiological transition of arousal, and could translate to a period of lower damage risk. It is possible that the longer amount of time to reach full arousal could have allowed enough time for the clearance of luzindole from the system, resulting in no difference between groups. It is also possible and likely that there are other protective mechanisms in place in the hypothalamus to help account for the missing melatonin receptor signaling.

Reportable Outcomes

Optimization of the BHB/M blood loss therapy

Our results show that a single 1 ml/kg bolus of BHB/M provides the same survival benefit as a bolus plus a slow infusion. In fact, animals that were administered a single bolus lived longer than those in which a slow infusion was continued. The lack of statistical difference could be the result of a small sample size. It is possible that a single bolus allows the organism to balance the osmolarity and electrolyte content of the blood. Continuing to infuse a hypertonic solution may exceed the individual's ability to compensate for the osmolar load and electrolyte imbalance (30).

We demonstrated that it is important to maintain the BHB concentration at 4M in order to obtain maximum survival, but melatonin can be administered at a concentration 10^6-fold lower than the previously published concentration (10) without affecting rate of survival. The sodium salt of BHB is responsible for both the fluid shift from the intracellular space into the intravascular space and as a carbon source that maintains ATP production. Consequently, 2 M BHB would account result in only half the plasma expansion and half the fuel source. Reducing the concentration by ten-fold would not even manage to increase plasma BHB above the

baseline circulating levels observed in our study and by Klein *et al* (10). Administering 4 M NaCl could not support survival as effectively as 4 M BHB because NaCl only has a circulatory benefit but does not fuel the production of ATP which is essential for the maintenance of normal cellular function.

Melatonin receptor signaling is important for optimal mitochondrial function

In addition to specifically protecting the hypothalamus during arousal from torpor, melatonin receptor signaling also appears to be important for optimal mitochondrial function in the whole brain during this time. Here, we report that disruption of melatonin receptor signaling during arousal from torpor results in decreased mitochondrial function, specifically including a significant decrease in the state 3 respiration rate of luzindole-treated animals when using succinate as a fuel. We also see a decrease in mitochondrial membrane potential with luzindole treatment, although this trend is not significant, potentially due to our small sample size. Melatonin is known to protect mitochondria (16), which is particularly evident in research in animal models of neurological disorders. Treatment with melatonin ameliorated mitochondrial dysfunction associated with Alzheimer's disease by restoring membrane potential and respiration rates in young mice (17). Luzindole partially blocked the ability of melatonin to restore mitochondrial function, indicating that melatonin receptors play a role in mitochondrial protection. Melatonin also slowed disease progression in a mouse model of Huntington's disease and this study also showed that melatonin receptor expression decreased in HD mice, suggesting that depletion of melatonin receptor signaling enhances the disease phenotype (18). Additionally, previous work has shown that melatonin protects mitochondria by antagonizing apoptotic signaling, as mentioned previously, an effect that is reversed by luzindole (19). Our work in the hibernator, which produces melatonin naturally during arousal from hibernation, supports this work by functionally demonstrating that disrupting melatonin receptor signaling upon arousal from torpor reduces mitochondrial function.

It is important to state that the effect of luzindole is seen in mitochondria isolated from the whole brain, not just the hypothalamus. It is possible that the effect of melatonin receptor

signaling on whole brain mitochondrial respiration and membrane potential is not necessarily just to protect the mitochondria, but to actually enhance and improve mitochondrial function during arousal in the brain, effectively allowing the hibernator's mitochondria to perform better than the mitochondria of a non-hibernating mammal in the same conditions. This idea is supported in part by the summer experiments, showing both that luzindole administration has no effect on mitochondrial function compared to vehicle and that the summer respiration rates and membrane potentials overall are lower than what is seen in hibernation (Figures 9-10). This is also supported by the ability of melatonin to restore mitochondrial function in a mouse model of Alzheimer's disease (17). Additionally, previous work showed that melatonin administration stimulated mitochondrial respiration in rat brain (16), however, it is not known whether this effect is receptor-mediated. Arousal is a very energy costly event requiring considerable energy generated by the mitochondria. It is possible that melatonin production upon arousal from torpor functions to help the brain mitochondria work harder and more efficiently during a period of extreme energy need.

Enhanced oxidative capacity of ground squirrel brain mitochondria during hibernation

During the hibernation season, thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) regularly cycle between bouts of torpor and interbout arousal (IBA). Most of the brain is electrically quiescent during torpor, but regains activity quickly upon arousal to IBA, resulting in extreme oscillations in energy demand during the hibernation season. We hypothesized that brain mitochondria undergo a seasonal change in function to accommodate the variable energy demands of hibernation. To address this hypothesis, we examined mitochondrial bioenergetics of the brain in thirteen-lined ground squirrels across two time points: hibernation (HIB) and spring (SP). Respiration rates of isolated brain mitochondria through complexes I and II of the electron transport system were significantly higher in HIB than in SP ($P < 0.05$). We also found a significant increase in membrane potential ($\Delta\psi_m$) between SP and HIB ($P < 0.05$), and proton leak was lower in HIB than in SP. Finally, there was a significant increase in calcium loading in SP brain mitochondria compared to HIB ($P < 0.001$). Overall, these data show that brain mitochondrial bioenergetics are not static across the year, and

suggest that brain mitochondria function more efficiently during the hibernation season, allowing for rapid production of energy in order to meet demand when extreme physiological changes are occurring. This study provides improved understanding of the overall energy requirements of a hibernator and characterizes the natural functional plasticity of brain mitochondria in hibernation.

Conclusion

There are three potential mechanisms by which BHB/M improves survival of hemorrhagic shock and they have immediate, short-term, and long-term effects. The immediate effects are unspecific and can be attributed to its osmolarity. It is well known that when administering a hypertonic solution into a blood vessel the changes in solute concentration will drive intracellular water into the intravascular space, expanding the plasma volume (20). This expansion can support an increase in MAP allowing blood to continue to circulate and minimizing ischemia.

In the short term, BHB/M action is twofold. First, BHB acts as fuel source preserving cell function. One of the main outcomes of hemorrhagic shock is adenosine triphosphate (ATP) depletion which leads to cell death (20). BHB dehydrogenase converts BHB into acetoacetate which binds CoA transferred from succinyl-CoA via succinyl-CoA transferase (SCOT). Acetoacetyl-CoA is subsequently converted into two acetyl-CoA molecules that can maintain ATP production via the TCA cycle. Hence, a single molecule of BHB is as energy efficient as a molecule of glucose without the potential of generating lactate. SCOT is the rate-limiting enzyme in this process and is also up-regulated in the heart of hibernating ground squirrels (21) during periods of torpor and low blood flow when BHB levels are elevated and glucose levels are depressed (22).

Second, melatonin is a powerful antioxidant and free radical scavenger. Reperfusion of an ischemic cell generates reactive oxygen species (ROS) which can damage cell membranes, directly injure DNA and proteins, and exacerbate inflammatory processes (23, 24), all of which

lead to apoptosis. Melatonin can neutralize ROS, minimizing reperfusion injury. Furthermore, the products of its reaction with free radicals, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), also possess antioxidant properties (25). The long term effects of BHB/M can also be attributed to melatonin as an immunomodulator, primarily through the scavenging of ROS and the inhibition of the activation of nuclear factor kappa B (NF-κB) (26). Limiting the inflammatory response in hemorrhagic shock and ischemia/reperfusion injury is paramount for long term survival. Patients who initially survive a hemorrhagic event could die weeks after the initial injury as the result of multiple organ failure (MOF) - a disproportionate self-destructive inflammation leading to the malfunction of organs not involved in the original traumatic event.

Neuroprotection against ROS Formation

Mammalian hibernators exhibit a compelling ability to tolerate oxidative stress during hibernation by eliminating free radicals generated during repeated cycles of torpor and arousal. Specifically, both glutathione and ascorbate play protective roles in the brain of ground squirrels during arousal form torpor (27-28). In addition, melatonin-receptor mediated mechanisms have been shown to aid in mitochondrial performance of thirteen-lined ground squirrel brain during arousal from torpor (29). Specifically, inhibition of melatonin receptor signaling reduced *state 3* respiration rates in brain mitochondria, thus suggesting melatonin aids in the mitochondrial performance of rapid production of ATP during arousal (29). It is possible that melatonin production upon arousal from torpor functions to help brain mitochondria work more efficiently during a period of extreme energy need (29).

So What?

Overall these experimental findings have resulted in three patents from the U.S. Patent Office over the course of this study (Patent No. 8,728,532, May 20, 2014; Patent No. 9,149,450, October 6, 2015; Patent No. 9,186,340, November 17, 2015). These findings will be presented at a Pre-IND Meeting of the FDA (PIND 130671) on July 8, 2016 with the ultimate goal of providing our warfighters with a portable low-volume therapy for severe blood loss.

We found that melatonin can be administered at concentrations a million-fold lower than the previously published formulation (10) and still support survival in the rat model. This is reasonable since serum melatonin peaks in rats are ~8.61×10^{-7} mM (31). Hence, administering a 1 ml/Kg bolus of 43 mM melatonin, the way it occurred in the experiments by Klein *et al* (10), would result in plasma levels almost fifty thousand times higher than peak. When 4 M BHB was administered without melatonin, initial survival was observed, but it could not be supported long term. This supports our assumption that long term survival is achieved through mechanisms involving the immunomodulatory properties of melatonin.

The anti-inflammatory properties of melatonin may be dose-dependent. Our histological results showed that the lowest dose of melatonin administered, 0.000043 mM, had statistically higher injury scores in small intestine than the one administered the highest dose of 4.3 mM in our second melatonin dose-ranging study. It is possible that other dose-dependent differences were masked by statistic variability and low sample size. Furthermore, the melatonin response observed at T_{30} in TNF-α levels also support the idea of a dose-dependent anti-inflammatory action of melatonin. However, these differences do not seem to affect survival, supporting a reduction in the concentration of melatonin in the composition of BHB/M in a rat model.

In summary, our experiments support an adjustment in the composition of the previously published BHB/M. The ketone component of BHB/M, beta-hydroxybutyrate, should remain at a concentration of 4 M. Melatonin can be administered at a concentration 10^6-fold lower than the previously published concentration (10) without affecting survival rate. Adjusting melatonin to a lower level has the benefit of reducing the concentration of the solvent DMSO. This is advantageous since there is some controversy over the use of DMSO (32). We also demonstrated that a slow infusion after a bolus administration is not necessary. This is a highly desirable trait as it increases the feasibility for self-administration in combat scenarios.

References

1. Kauvar DS, Wade CE. The epidemiology and modern management of traumatic hemorrhage: US and international perspectives. Critical Care. 2005;9(Suppl 5):S1-S9.

2. Rhee P, Alam H, Ling G. Hemorrhagic Shock and Resuscitation: Trauma Research at the Trauma Research and Readiness Institute for Sugery. In: Tsokos G, Atkins J, editors. Combat Medicine: Basic and Clinical Research in Military, Trauma, and Emergency Medicine. Totowa, New Hersey: Humana Press Inc.; 2003.

3. Support ACoSCoTSoATL. Advanced Trauma Life Support Course for Physicians: The Committee; 1989.

4. Alam HB, Koustova E, Rhee P. Combat Casualty Care Research: From Bench to the Battlefield. World Journal of Surgery. 2005;29(0):S7-S11.

5. Geiser F, Mzilikazi N. Does torpor of elephant shrews differ from that of other heterothermic mammals? Journal of Mammalogy. 2011;92(2):452-9.

6. D'Alecy L. Beta-hydroxybutyrate and response to hypoxia in the ground squirrel, Spermophilus tridecemlineatus. Comparative Biochemistry and Physiology B. 1990;96(1):189-93.

7. Andrews MT. Advances in molecular biology of hibernation in mammals. Bioessays. 2007;29(5):431-40.

8. Graf R, Schaller B. "Natural" Tolerance in Hibernators: Can We Learn from Physiological Preconditioning Against Ischemic or Hypoxic Brain Injury? In: Schaller B, editor. Cerebral Ischemic Tolerance: From Animal Models to Clinical Relevance. Hauppauge, New York: Nova Science Publishers, Inc.; 2004.

9. Frerichs K, Kennedy C, Sokoloff L, Hallenbeck J. Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia". *J Cerebr Blood F Met* 14: 193-205, 1994.

10. Klein AH, Wendroth SM, Drewes LR, Andrews MT. Small-Volume d-[beta]-Hydroxybutyrate Solution Infusion Increases Survivability of Lethal Hemorrhagic Shock in Rats. Shock. 2010;34(6):565-72.

11. Mulier KE, Lexcen DR, Luzcek E, Greenberg JJ, Beilman GJ. Treatment with beta-hydroxybutyrate and melatonin is associated with improved survival in a porcine model of hemorrhagic shock. Resuscitation. 2012;83(2):253-8.

12. Carey LC, Lowery BD, Cloutier CT. Hemorrhagic shock. Current problems in surgery. 1971;8(1):1-48.

13. Radogna F, Paternoster L, Albertini MC, Cerella C, Accorsi A, Bucchini A, Spadoni G, Diamantini G, Tarzia G, Nicola MD. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. *J Pineal Res* 43: 154-162, 2007.

14. Radogna F, Cristofanon S, Paternoster L, D'Alessio M, De Nicola M, Cerella C, Dicato M, Diederich M, Ghibelli L. Melatonin antagonizes the intrinsic pathway of apoptosis via mitochondrial targeting of Bcl-2. *J Pineal Res* 44: 316-325, 2008.

15. Wang Z, Liu D, Wang J, Liu S, Gao M, Ling EA, Hao A. Cytoprotective effects of melatonin on astroglial cells subjected to palmitic acid treatment in vitro. *J Pineal Res* 52: 253-264, 2012.

16. Acuña-Castroviejo D, Martín M, Macías M, Escames G, León J, Khaldy H, Reiter RJ. Melatonin, mitochondria, and cellular bioenergetics. *J Pineal Res* 30: 65-74, 2001.

17. Dragicevic N, Copes N, O'Neal-Moffitt G, Jin J, Buzzeo R, Mamcarz M, Tan J, Cao C, Olcese JM, Arendash GW. Melatonin treatment restores mitochondrial function in Alzheimer's mice: a mitochondrial protective role of melatonin membrane receptor signaling. *J Pineal Res* 51: 75-86, 2011.

18. Wang X, Sirianni A, Pei Z, Cormier K, Smith K, Jiang J, Zhou S, Wang H, Zhao R, Yano H. The melatonin MT1 receptor axis modulates mutant Huntingtin-mediated toxicity. *J Neurosci* 31: 14496-14507, 2011.

19. Wang Z, Liu D, Wang J, Liu S, Gao M, Ling EA, Hao A. Cytoprotective effects of melatonin on astroglial cells subjected to palmitic acid treatment in vitro. *J Pineal Res* 52: 253-264, 2012.

20. Kashiwaya Y, Takeshima T, Mori N, Nakashima K, Clarke K, Veech RL. d-β-Hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. Proceedings of the National Academy of Sciences. 2000;97(10):5440-4.

21. Russeth, KP, Higgins, L, and Andrews, MT. Identification of proteins from non-model organisms using mass spectrometry: Application to a hibernating mammal. J. Proteome Res. 2006; 5, 829-839.

22. Andrews, MT, Russeth, KP, Drewes, LR, and Henry, PG Adaptive mechanisms regulate preferred utilization of ketones in the heart and brain of a hibernating mammal during arousal from torpor. 2009; Am. J. Physiol. 296, R383-393.

23. Sanada S, Komuro I, Kitakaze M. Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures. American Journal of Physiology-Heart and Circulatory Physiology. 2011;301(5):H1723-H41.

24. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. The FASEB Journal. 2003;17(10):1195-214.

25. Mayo JC, Sainz RM, Tan D-X, Hardeland R, Leon J, Rodriguez C, et al. Anti-inflammatory actions of melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), in macrophages. Journal of neuroimmunology. 2005;165(1):139-49.

26. Cuzzocrea S, Reiter RJ. Pharmacological action of melatonin in shock, inflammation and ischemia/reperfusion injury. European journal of pharmacology. 2001;426(1):1-10.

27. Dave, K. R., Christian, S. L., Perez-Pinzon, M. A. and Drew, K. L. (2012). Neuroprotection : Lessons from hibernators. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 162, 1–9.

28. Drew, K. L., Osborne, P. G., Frerichs, K. U., Hu, Y., Koren, R. E., Hallenbeck, J. M. and Rice, M. E. (1999). Ascorbate and glutathione regulation in hibernating ground squirrels. *Brain Res.* 851, 1–8.

29. Schwartz, C., Ballinger, M.A. and Andrews, M.T. (2015). Melatonin receptor signaling contributes to neuroprotection upon arousal from torpor in the thirteen-lined ground squirrel. *Am. J. Phys. Regul. Integ. Comp. Phys* 309: R1292-R1300.

30. Kreimeier U, Messmer K. Small‐volume resuscitation: from experimental evidence to clinical routine. Advantages and disadvantages of hypertonic solutions. Acta anaesthesiologica scandinavica. 2002;46(6):625-38.

31. Benot S, Molinero P, Soutto M, Goberna R, Guerrero JM. Circadian variations in the rat serum total antioxidant status: Correlation with melatonin levels. Journal of Pineal Research. 1998;25(1):1-4.

32. Davis PW. An Incipient "Wonder Drug" Movement: DMSO and the Food and Drug Administration. Social Problems. 1984:197-212.

Appendices

Tables

Table 1. Melatonin Dose-Ranging Study I: Mean survival time in animals subjected to 60% blood loss at 24 hours and ten days.

Treatment		Mean ± SD	
		24 Hrs	10 Days
4.3 mM Mel		21.00 ± 2.74	7.375 ± 1.75
43 mM Mel		21.00 ± 2.74	6.375 ± 2.00
Sham		24.00	10.00
Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
4.3 mM Mel	43 mM Mel	1.0000	0.7980
4.3 mM Mel	Sham	0.1760	0.0143
43 mM Mel	Sham	0.1760	0.0143

Mean survival time calculated as the area under the Kaplan-Meier curve. Units are hours for the calculations at 24 hours and days for the calculations at ten days. The treatment labeled as 43 mM Mel contains 4 M BHB with 43 mM melatonin in 20% DMSO (n=6); the treatment labeled as 4.3 mM Mel contains 4 M BHB with 4.3 mM melatonin in 10% DMSO (n=6). Statistically significant *p*-values are colored in red and underlined. Abbreviations: Mel, melatonin.

Table 2. BHB Dose-Ranging Study: Mean survival time in animals subjected to 60% blood loss at 24 hours and ten days.

Treatment		Mean ± SD	
		24 Hrs	10 Days
0.4 M BHB		6.50 ± 4.64	20.07 ± 2.05
2 M BHB		20.40 ± 3.22	5.25 ± 2.21
4 M BHB		21.00 ± 2.74	7.38 ± 1.75
Sham		24.00	10.00
Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
0.4 M BHB	2 M BHB	0.0398	0.0803
0.4 M BHB	4 M BHB	0.0222	0.0472
0.4 M BHB	Sham	0.0004	0.0004
2 M BHB	4 M BHB	0.8920	0.4990
2 M BHB	Sham	0.1380	0.0041
4 M BHB	Sham	0.1760	0.0102

Mean survival time calculated as the area under the Kaplan-Meier curve. Units are hours for the calculations at 24 hours and days for the calculations at ten days. Sample sizes are as follows: 4 M BHB, n=6; 2 M BHB, n=5; 0.4 M BHB, n=5. All solutions contained 4.3 mM melatonin and 10% DMSO. Statistically significant *p*-values are colored in red and underlined. Abbreviations: BHB, D-stereoisomer of beta-hydroxybutyrate.

Table 3. Melatonin Dose-Ranging Study II: Mean survival time in animals subjected to 60% blood loss at 24 hours and ten days.

Treatment	Mean ± SD	
	24 Hrs	10 Days
0 mM Mel	18.6 ± 3.20	4.38 ± 1.42
0.000043 mM Mel	21.71 ± 2.76	7.71 ± 1.10
0.0043 mM Mel	18.60 ± 3.20	3.58 ± 1.60
.43 mM Mel	22.20 ± 2.30	7.63 ± 1.44
4.3 mM Mel	20.40 ± 3.22	7.75 ± 1.49
NaCl Control	18.60 ± 3.20	4.58 ± 1.42
Sham	24	10.00

Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
Sham	0 mM Mel	0.0555	0.0009
Sham	4.3 mM Mel	0.1280	0.0555
Sham	.43 mM Mel	0.2940	0.0555
Sham	0.0043 mM Mel	0.0555	0.0229
Sham	0.000043 mM Mel	0.2940	0.0555
Sham	NaCl Control	0.0555	0.0009
0 mM Mel	4.3 mM Mel	0.6150	0.1200
0 mM Mel	.43 mM Mel	0.2760	0.0792
0 mM Mel	0.0043 mM Mel	1.0000	0.3560
0 mM Mel	0.000043 mM Mel	0.3570	0.0864
0 mM Mel	NaCl Control	1.0000	0.9080
4.3 mM Mel	.43 mM Mel	0.5420	0.9630
4.3 mM Mel	0.0043 mM Mel	0.6150	0.5910
4.3 mM Mel	0.000043 mM Mel	0.6260	0.9630
4.3 mM Mel	NaCl Control	0.6150	0.1310
.43 mM Mel	0.0043 mM Mel	0.2760	0.5630
.43 mM Mel	0.000043 mM Mel	0.9420	1.0000
.43 mM Mel	NaCl Control	0.2760	0.0866
0.0043 mM Mel	0.000043 mM Mel	0.3570	0.6570
0.0043 mM Mel	NaCl Control	1.0000	0.3550
0.000043 mM Mel	NaCl Control	0.3570	0.0866

Mean survival time calculated as the area under the Kaplan-Meier curve. Units are hours for the calculations at 24 hours and days for the calculations at ten days. Sample sizes are as follows: 4.3 mM melatonin, n=10; 0.43 mM melatonin, n=10; 0.0043 mM melatonin, n=10); 0.000043 mM melatonin, n=10; and 0 mM melatonin, n=10. These solutions contained 4 M BHB and 2% DMSO. A control group was also included and were administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=10). Statistically significant *p*-values are colored in red and underlined. Abbreviations: Mel, melatonin.

FIGURE LEGENDS

Figure 1. Optimization of delivery experiments: Experimental timeline. After surgical preparation, animals were hemorrhaged until MAP ~25mmHg. They were then infused with either (A) a single 1 ml/kg bolus, or (B) a bolus followed by a 100 µl/hr slow infusion. In both instances, the bolus infusion was administered within 10 minutes of achieving MAP ~25mmHg. After bolus administration, animals were further hemorrhaged to 60% of their calculated blood volume. No blood was transfused at any time point. Animals were monitored for 24 hours. All 24-hour survivors were euthanized.

Figure 2. Optimization of delivery experiments: Kaplan-Meier plot of animals subjected to 60% blood loss. Infusion of the published formulation of BHB/M (4 M BHB with 43 mM melatonin in 20% DMSO) was conducted by administering either a single 1 ml/kg bolus (n=6) or a bolus followed by a 100 µl/hr slow infusion (n=5). The groups were not statistically different from each other. Times on the x-axis reflect minutes after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Figure 3. Optimization of composition experiments: Experimental timeline. After surgical preparation, animals were hemorrhaged until MAP ~25mmHg and infused with a single 1 ml/kg bolus of solution over a 10 minute period. After bolus administration, animals were further hemorrhaged to 60% of their calculated blood volume and maintained in a shocked state for one hour. One-half (50%) of the shed blood volume was auto-transfused at a rate of 500 µl/min 60 minutes after achieving 60% blood loss. Animals were monitored for 10 days. All animals surviving 10 days were euthanized.

Figure 4. Melatonin Dose-Ranging Study I: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. Infusion of either 4 M BHB with 43 mM melatonin in 20% DMSO (n=6) or 4 M BHB with 4.3 mM melatonin in 10% DMSO (n=6) was achieved by administering a single 1 ml/kg bolus. Sham-operated animals are also included in the graph. Times on the x-axis reflect either hours (panel A) or days (panel B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Figure 5. BHB Dose-Ranging Study: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. Infusion of either 4 M BHB (n=6), 2 M BHB (n=5), or 0.4 M BHB (n=5) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4.3 mM melatonin and 10% DMSO. Sham-operated animals are also included in the graph. Times on the x-axis reflect either hours (A) or days (B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Figure 6. Melatonin Dose-Ranging Study II: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. Infusion of either 4.3 mM melatonin (n=10), 0.43 mM melatonin (n=10), 0.0043 mM melatonin (n=10), 0.000043 mM melatonin (n=10), or 0 mM melatonin (n=10) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4 M BHB and 2% DMSO. A control group was administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=10). Sham-operated animals are also included in the graph. The time shown on the x-axis reflect either hours (panel A) or days (panel B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Figure 7. Caspase-3 activity in ground squirrel brain. Caspase-3 activity (µmol pNA) at the mid-arousal endpoint after vehicle (black) or luzindole (gray) treatment in cortex, brainstem, hippocampus, and hypothalamus (20 and 37°C). Luzindole-treated animals had significantly higher caspase-3 activity in the hypothalamus at the 20°C endpoint (*T-test, *P*=0.01, N=5 per group). Error bars represent standard error of the mean. pNA: p-nitroaniline.

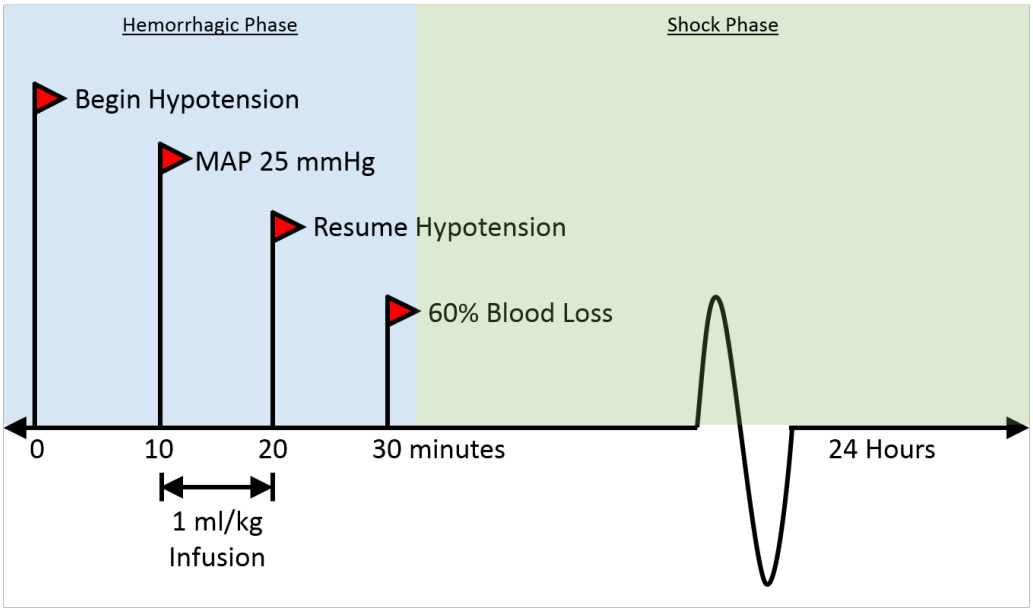
Figure 8. Expression of total STAT3 and phosphorylated STAT3 [Y705] in ground squirrel brain. There was no significant difference between groups in total STAT3 (A) or P-STAT3 [Y705] (B) (N=5 per group). The bar graphs represent quantification of western blots. Error bars represent standard error of the mean.

Figure 9. State 3 respiration rates in brain mitochondria. A. State 3 respiration rates at mid-arousal from hibernation in brain mitochondria from animals treated with luzindole (gray) or vehicle (black). Luzindole-treated animals had significantly lower respiration rates with succinate (*T-test, *P*=0.019). B. State 3 respiration rates during summer in brain mitochondria from animals treated with luzindole (gray) or vehicle (black). Vehicle-treated animals had

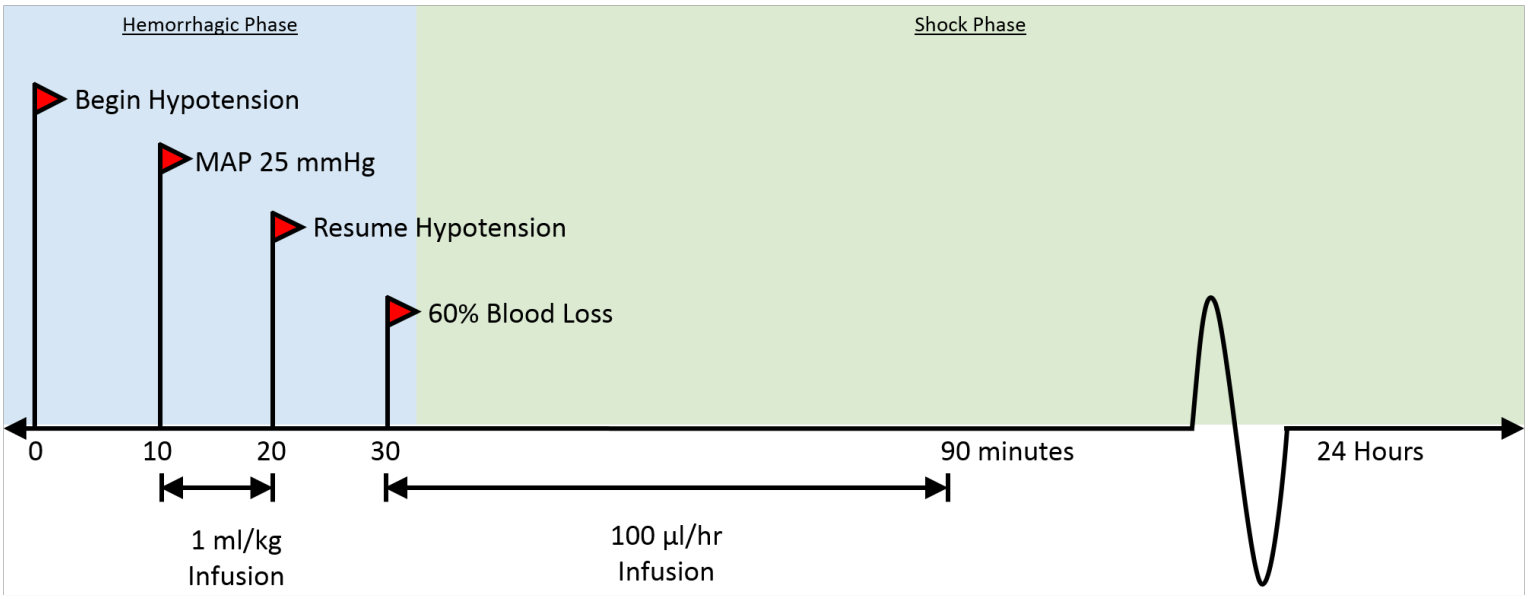
significantly higher respiration rates with G3P (*T-test, *P*=0.0496). Number of animals (N) for each group is listed at the bottom of the respective bar. Error bars represent standard error of the mean. G/M: glutamate/malate, SUC: succinate, G3P: glycerol-3-phosphate

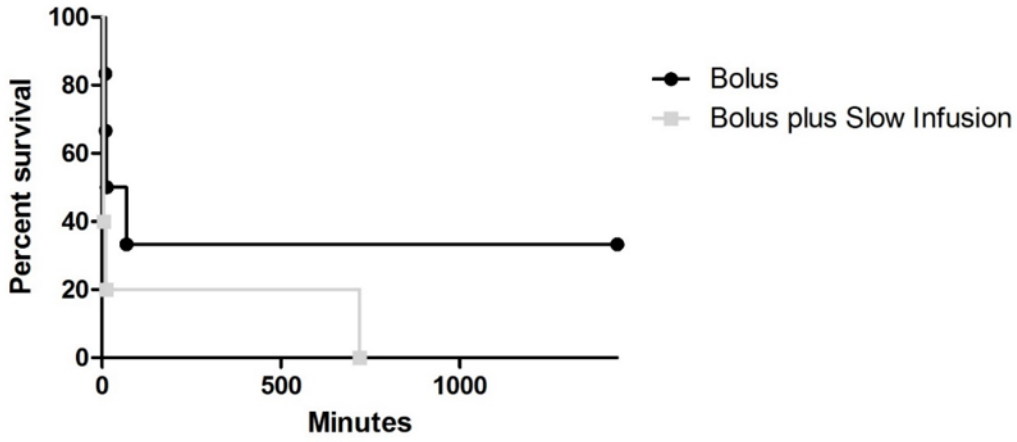
Figure 10. Kinetic response of proton leak according to mitochondrial membrane potential ($\Delta\Psi_m$) in ground squirrel brain mitochondria during hibernation (triangles) and summer (circles). Luzindole-treated animals are shown in black and vehicle-treated animals are shown in gray (N=5 per group). No significant effect of treatment is seen in either season, but luzindole-treated animals tended to have lower membrane potential during hibernation. Error bars were omitted for clarity. VEH: vehicle, LUZ: luzindole.

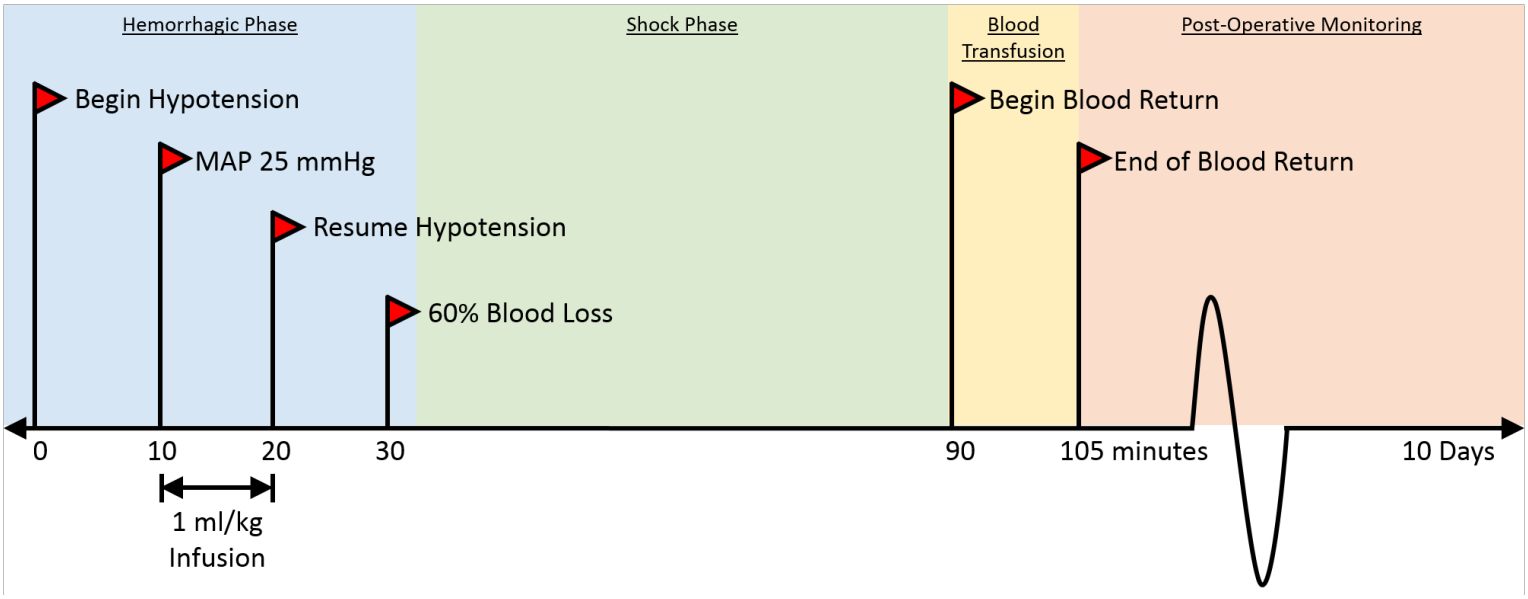
A


Hemorrhagic Phase
Shock Phase
Begin Hypotension
MAP 25 mmHg
Resume Hypotension
60% Blood Loss
0
10
20
30 minutes
24 Hours
1 ml/kg
Infusion

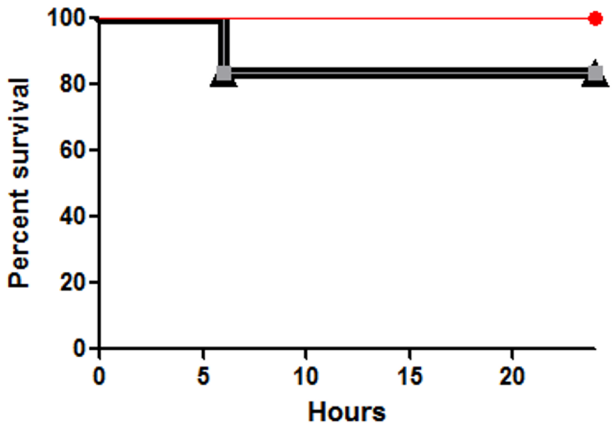
B


Hemorrhagic Phase
Shock Phase
Begin Hypotension
MAP 25 mmHg
Resume Hypotension
60% Blood Loss
0
10
20
30
90 minutes
24 Hours
1 ml/kg
Infusion
100 µl/hr
Infusion

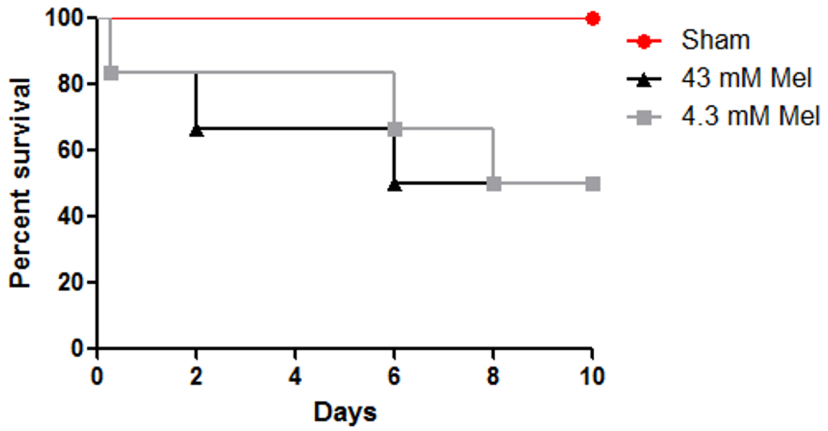

100
80
60
40
20
0
Percent survival
0
500
1000
Minutes
Bolus
Bolus plus Slow Infusion


Hemorrhagic Phase
Shock Phase
Blood Transfusion
Post-Operative Monitoring
Begin Hypotension
MAP 25 mmHg
Resume Hypotension
60% Blood Loss
Begin Blood Return
End of Blood Return
0
10
20
30
90
105 minutes
10 Days
1 ml/kg Infusion

A


Percent survival
100
80
60
40
20
0
0
5
10
15
20
Hours

B


Percent survival
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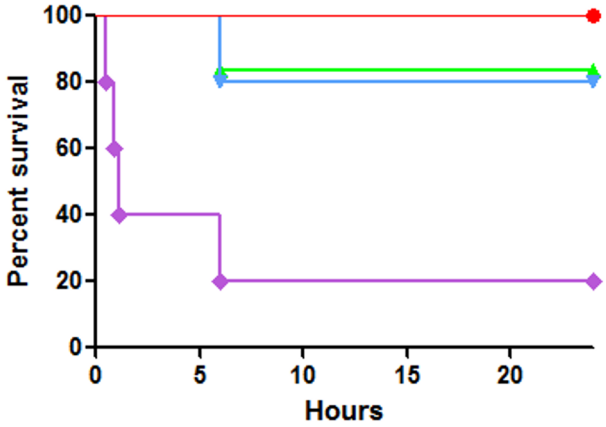
80
60
40
20
0
0
2
4
6
8
10
Days
Sham
43 mM Mel
4.3 mM Mel

A


Percent survival
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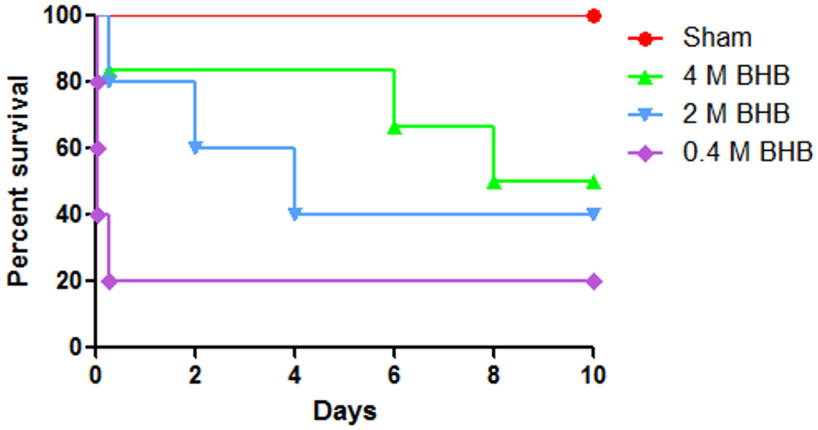
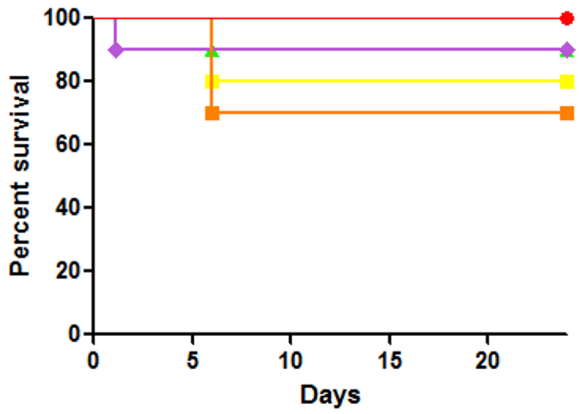
80
60
40
20
0
0
5
10
15
20
Hours

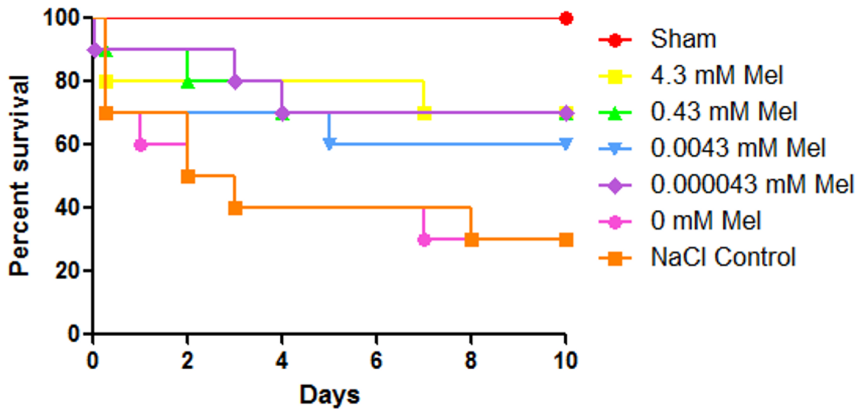
B

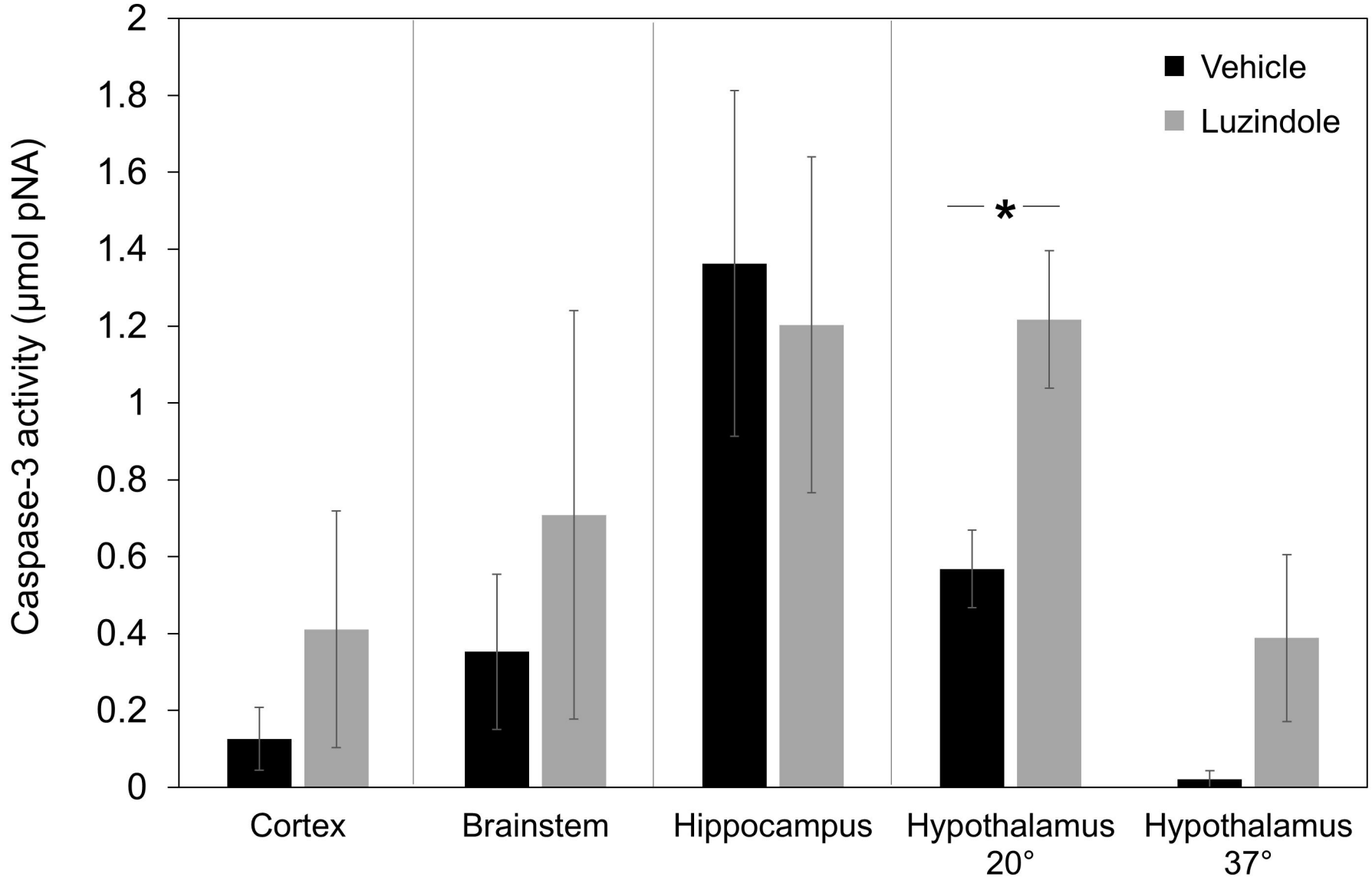

Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
Days
Sham
4 M BHB
2 M BHB
0.4 M BHB

A

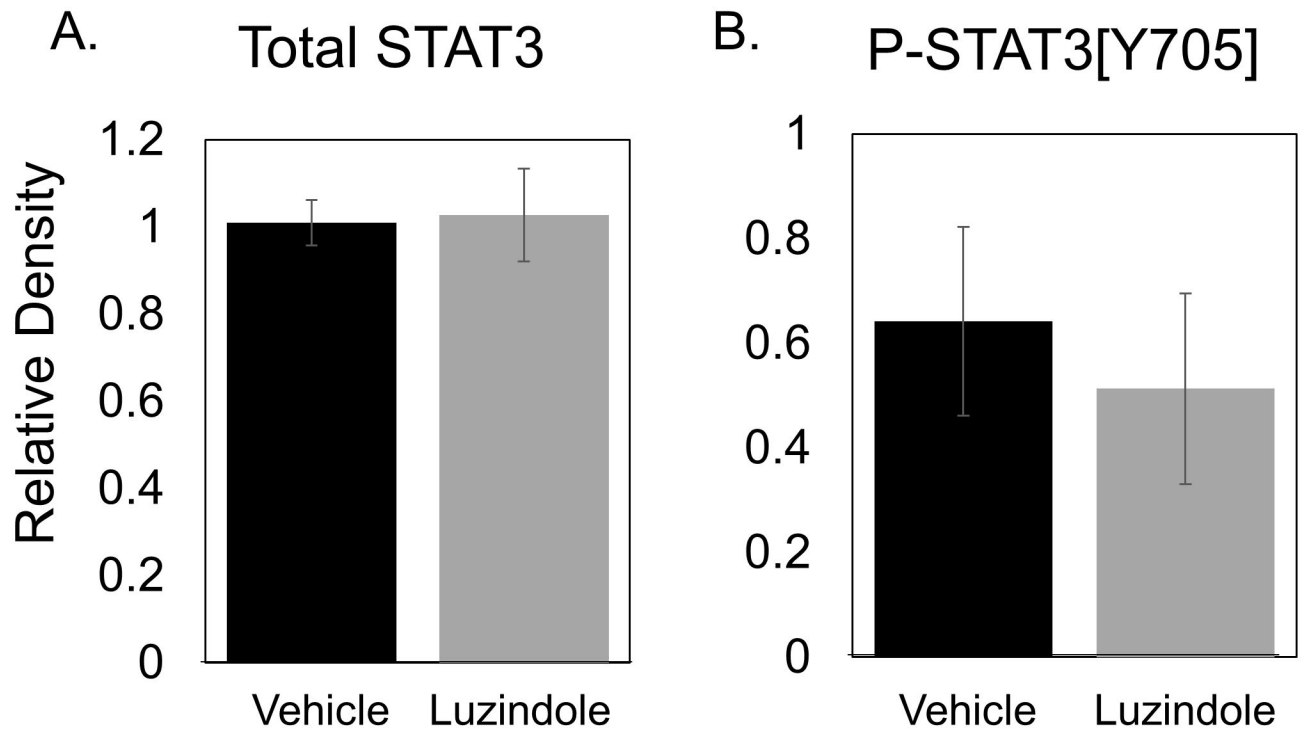

Percent survival
100
80
60
40
20
0
0
5
10
15
20
Days

B

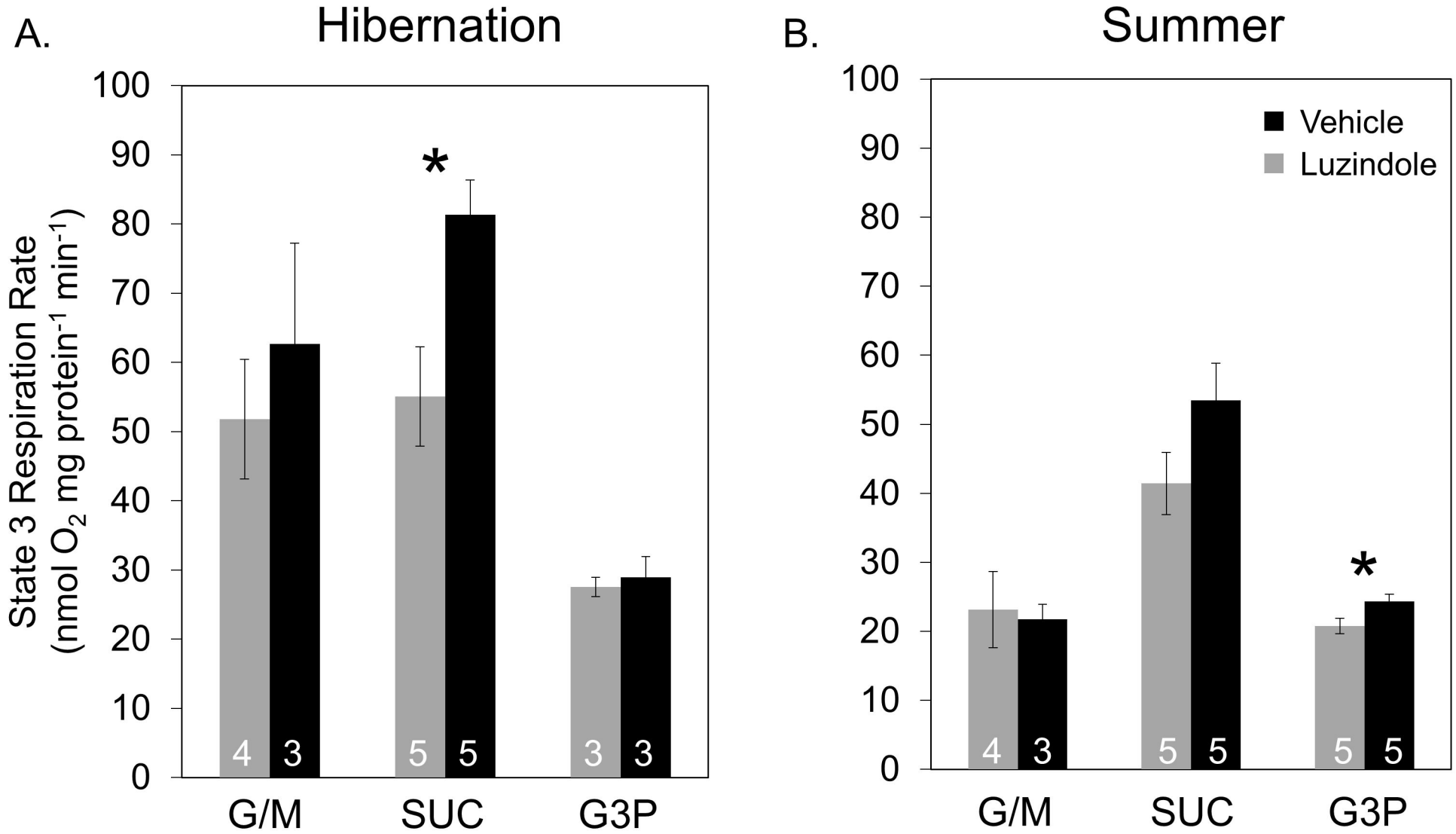

Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
Days
Sham
4.3 mM Mel
0.43 mM Mel
0.0043 mM Mel
0.000043 mM Mel
0 mM Mel
NaCl Control



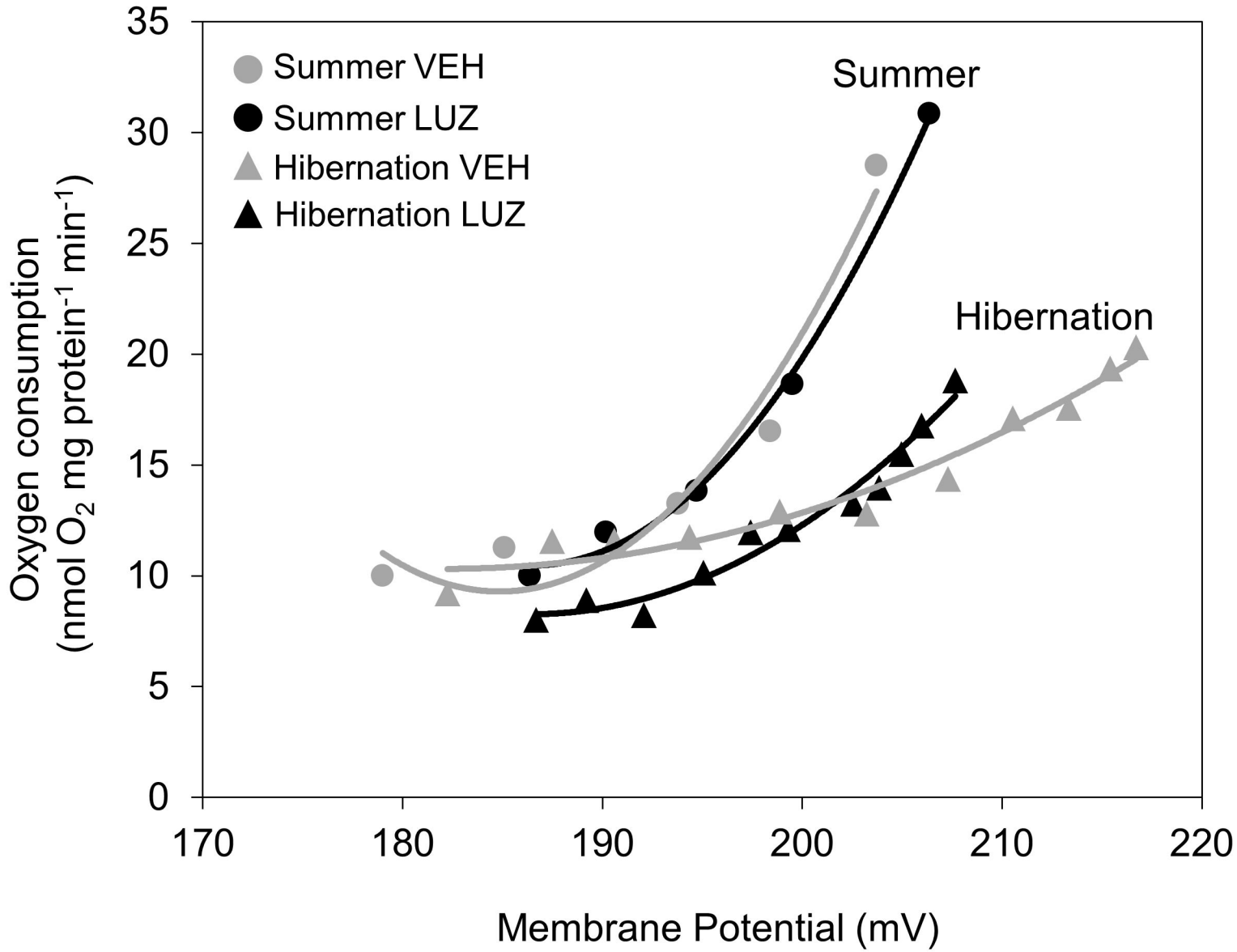
Schwartz, Ballinger, and Andrews, Figure 3



Schwartz, Ballinger, and Andrews, Figure 4



Schwartz, Ballinger, and Andrews, Figure 5



Schwartz, Ballinger, and Andrews, Figure 6